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Development of Nested PCR Based on the ViaB Sequence To Detect *Salmonella typhi*

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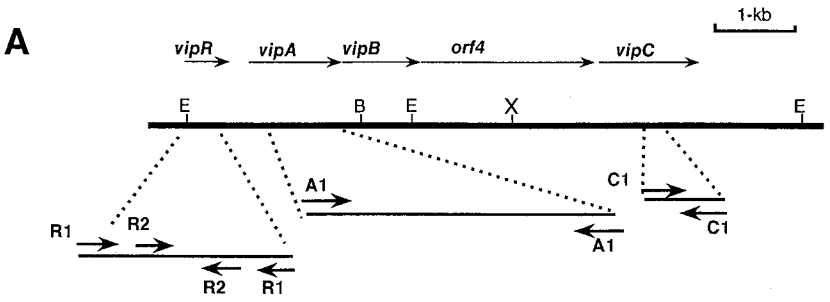
For a rapid diagnosis of typhoid fever, we developed a nested PCR based on the nucleotide sequence encoding the Vi antigen. All *Salmonella typhi* strains along with a *Salmonella paratyphi* C strain were PCR positive. This assay was able to detect *S. typhi* at the single-cell level.

Typhoid fever is still one of the most important infectious diseases worldwide. The conventional culture method for detecting *Salmonella typhi* in clinical samples takes 2 to 5 days and sometimes takes more than 1 week with blood samples. Many successful approaches for rapidly detecting *S. typhi* have been reported (for example, detection of typhoid antibody or antigen and by DNA probe assay), but these methods are limited by poor sensitivity. Recently, a nested PCR based on the *H1-d* flagellin gene was reported to detect *S. typhi* in blood samples (2). It demonstrated that a nested PCR assay is sensitive enough to detect *S. typhi* directly from blood specimens. However, this assay is not applicable to the clinical specimens in which salmonella serovars are frequently present (for example, stool specimens), as the possibility that it may also detect other salmonella serovars with the *H1-d* flagellin gene exists.

All *S. typhi* strains isolated from blood specimens possess the Vi antigen, an antigen associated with virulence. The Vi antigen occurs in only a few organisms. *S. typhi* strains, *Salmonella paratyphi* C strains, and a few strains of *Citrobacter freundii* and *Salmonella dublin* are the only organisms having the Vi antigen. Thus, the DNA sequence encoding the Vi antigen (called the ViaB region) is useful in developing DNA-based diagnostic tests for *S. typhi*. In this study, we designed several PCR primer pairs based on the ViaB sequence and developed a nested PCR for rapidly and specifically detecting *S. typhi* in clinical specimens.

We designed four PCR primer sets (R1, R2, C1, and A1) from the nucleotide sequence encoding Vi antigen (1) (GenBank accession no. D14156). The location of primers on the physical map of the ViaB region and the primer sequence are shown in Fig. 1. For the nested PCR, R1 and R2 primers were used for first and second PCRs, respectively. R1 (5′-GTTATTTCAGCATAAGGAG-3′ and 5′-ACTTGTCCGTGTTTTACTC-3′) and R2 (5′-GTGAACCTAAATCGCTACAG-3′ and 5′-CTTCCATACCACTTTCCG-3′) amplified 599 bp of DNA (nucleotides 745 to 1343) and 307 bp of DNA (nucleotides 877 to 1183), respectively.

A bacterial suspension containing 10^8 CFU/ml was prepared, and DNA was extracted as described by Yamamoto et al. (3). One milliliter of final preparation contained DNA ex-



B

	Forward	Reverse	Size of product
R1	5'-GTTATTTCAGCATAAGGAG-3'	5'-ACTTGTCCGTGTTTTACTC-3'	599-bp
R2	5'-GTGAACCTAAATCGCTACAG-3'	5'-CTTCCATACCACTTTCCG-3'	307-bp
A1	5'-CCTACTTCGAAAGTCGAC-3'	5'-CACGAATTGATATATCGCCT-3'	1221-bp
C1	5'-TCAATCTTGACCAGGCTTGC-3'	5'-TAACGAGGCTGAGAGGCAAT-3'	160-bp

FIG. 1. Restriction map of ViaB region showing positions (A) and sequences (B) of six oligonucleotide primers. (B). For the nested PCR, R1 and R2 primers were used for the first and second PCRs, respectively. The translocation region localized within the ViaB region is not shown. B, *Bam*HI; E, *Eco*RV; X, *Xho*I.

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TABLE 1. Results of PCR based on the ViaB sequence

GIFU no.	Organism	Serogroup	Vi antigen	Result of PCR with following primer pairs:			
				A1	C1	R1	R1 + R2[a]
12697	*Salmonella paratyphi* A	A	−	ND[b]	ND	−	−
3163	*Salmonella typhimurium*	B	−	ND	ND	−	−
12695	*S. paratyphi* B	B	−	ND	ND	−	−
12814	*Salmonella essen*	B	−	ND	ND	−	−
12825	*Salmonella reading*	B	−	ND	ND	−	−
2919	*Salmonella choleraesuis*	C	−	ND	ND	−	−
12698	*Salmonella oranienburg*	C	−	ND	ND	−	−
12817	*Salmonella lomita*	C	−	ND	ND	−	−
12823	*S. paratyphi* C	C	+	+	+	+	+
12819	*Salmonella newport*	C	−	ND	ND	−	−
12789	*Salmonella muenchen*	C	−	ND	ND	−	−
12829	*Salmonella thompson*	C	−	ND	ND	−	−
12816	*Salmonella kentucky*	C	−	ND	ND	−	−
12830	*Salmonella virginia*	C	−	ND	ND	−	−
3161	*Salmonella enteritidis*	D	−	ND	ND	−	−
2922	*Salmonella typhi*	D	+	ND	ND	+	+
10003	*S. typhi*	D	+	ND	ND	+	+
10004	*S. typhi*	D	+	ND	ND	+	+
10005	*S. typhi*	D	+	ND	ND	+	+
10006	*S. typhi*	D	+	ND	ND	+	+
10007	*S. typhi*	D	+	+	+	+	+
12686	*S. typhi*	D	+	ND	ND	+	+
12699	*Salmonella dublin*	D	−	ND	ND	−	−
13000	*S. dublin*	D	+	+	+	−	−
13011	*S. dublin*	D	+	+	+	−	−
13012	*S. dublin*	D	+	+	+	−	−
12815	*Salmonella gallinarum*	D	−	ND	ND	−	−
12822	*Salmonella panama*	D	−	ND	ND	−	−
12824	*Salmonella pullorum*	D	−	ND	ND	−	−
12826	*Salmonella sendai*	D	−	ND	ND	−	−
12828	*Salmonella strasbourg*	D	−	ND	ND	−	−
12818	*Salmonella london*	E	−	ND	ND	−	−
12813	*Salmonella anatum*	E	−	ND	ND	−	−
12787	*Salmonella liverpool*	E	−	ND	ND	−	−
12827	*Salmonella senftenberg*	E	−	ND	ND	−	−
12831	*Salmonella westerstede*	E	−	ND	ND	−	−
12694	*Salmonella aberdeen*	F	−	ND	ND	−	−
12834	*Salmonella poona*	G	−	ND	ND	−	−
12835	*Salmonella worthington*	G	−	ND	ND	−	−
12821	*Salmonella onderstepoort*	H	−	ND	ND	−	−
12833	*Salmonella cerro*	K	−	ND	ND	−	−
12832	*Salmonella champaign*	Q	−	ND	ND	−	−
12700	*Salmonella nairobi*	T	−	ND	ND	−	−
12820	*Salmonella niarembe*	V	−	ND	ND	−	−
8666	*Staphylococcus aureus*		−	ND	ND	−	−
8101	*Streptococcus pyogenes*		−	ND	ND	−	−
8766	*Streptococcus pneumoniae*		−	ND	ND	−	−
3178	*Neisseria meningitidis*		−	ND	ND	−	−
2925	*Citrobacter freundii*		−	ND	ND	−	−
12284	*C. freundii*		+	−	+	−	−
2926	*Enterobacter cloacae*		−	ND	ND	−	−
12490	*Escherichia coli*		−	ND	ND	−	−
10424	*Haemophilus influenzae*		−	ND	ND	−	−
2924	*Klebsiella pneumoniae*		−	ND	ND	−	−
274	*Pseudomonas aeruginosa*		−	ND	ND	−	−
2916	*Shigella dysenteriae*		−	ND	ND	−	−
8908	*Shigella flexneri*		−	ND	ND	−	−
4870	*Shigella sonnei*		−	ND	ND	−	−
1323	*Vibrio cholerae*		−	ND	ND	−	−
1339	*Vibrio parahaemolyticus*		−	ND	ND	−	−
3138	*Yersinia enterocolitica*		−	ND	ND	−	−
3035	*Yersinia pseudotuberculosis*		−	ND	ND	−	−

[a] One microliter of the PCR product amplified with R1 primers was used as a sample for a second PCR with R2 primers.
[b] ND, not done.

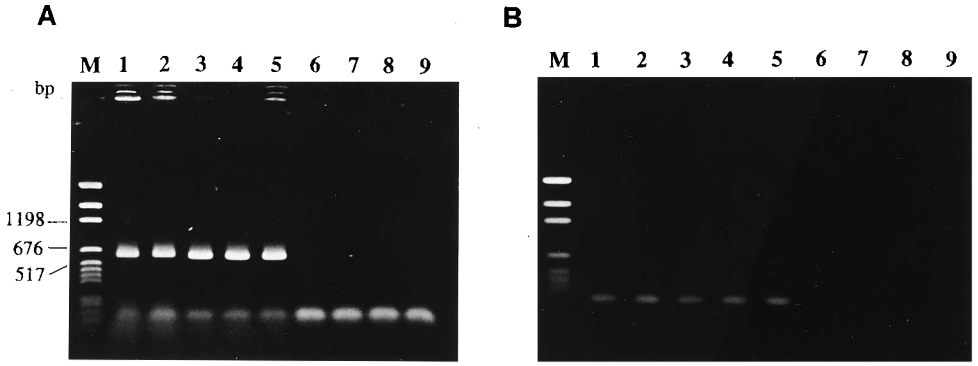

FIG. 2. Specificities of the first (A) and second (B) PCRs for Vi-positive organisms. Lane M, pGEM DNA markers (Seikagaku Corporation, Tokyo, Japan); lane 1, *S. typhi* GIFU2922; lane 2, *S. typhi* GIFU10004; lane 3, *S. typhi* GIFU10007; lane 4, *S. typhi* GIFU12686; lane 5, *S. paratyphi* C GIFU1283; lane 6, *C. freundii* GIFU12284; lane 7, *S. dublin* GIFU13000; lane 8, *S. dublin* GIFU13011; lane 9, *S. dublin* GIFU13012.

tracted from 10^8 cells. These samples were stored at −80°C until PCR was performed. The first PCR was carried out with a 100-μl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2 U of recombinant *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μM each deoxynucleoside triphosphate, primers (0.6 μM each), and 10 μl of the sample. The PCR was carried out in a DNA Thermal Cycler Model 321 (Perkin-Elmer Cetus). The temperature cycling was as follows: 35 cycles were done at 96°C for 30 s, 56°C for 15 s, and 74°C for 30 s. The temperature was maintained at 96°C for 3 min at the start of the first cycle and at 74°C for 10 min at the end of the last cycle. In every PCR experiment, saline was treated as described above and used as a negative control. The second PCR, with R2 primers, was performed as follows: 1 μl of the first PCR product amplified with R1 primers was transferred to the second reaction mixture, which contained 0.6 μM (each) R2 primers and the buffer that was used in the first PCR. The temperature cycling of the second PCR was the same as in the first PCR. The amplified PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination.

At first, to select more specific primers for *S. typhi*, we examined the specificities of three PCR primer pairs (R1, A1, and C1) for Vi-positive strains of *S. typhi*, *S. paratyphi* C, *S. dublin*, and *C. freundii*. The results are shown in Table 1. The R1 primer pair was more specific than other primer pairs. This primer pair amplified DNA only from *S. typhi* and *S. paratyphi* C. Thus, the R1 primer pair was used for constructing the nested PCR amplification. C1 primers amplified DNA from all six Vi-positive strains tested. This primer pair may be specific for ViaB regions regardless of species and serovars. A1 primers amplified DNA from all five Vi-positive salmonellae tested but did not amplify DNA from one Vi-positive *C. freundii* strain. This primer pair may be specific for all Vi-positive salmonellae. The primers described here may provide epidemiological tools for differentiating or confirming Vi-positive organisms.

Next, we constructed a nested PCR with R1 and R2 primer pairs and examined its specificity and sensitivity. We analyzed 44 strains of salmonellae and 18 strains of other genera for specificity. The first PCR with R1 primers amplified the DNA from all *S. typhi* strains and only one *S. paratyphi* C strain (Table 1). A band migrating close to the predicted 599-bp size was detected by agarose gel electrophoresis and ethidium bromide staining (Fig. 2A). The predicted amplified DNA was not detected in other strains. The specificity of the nested PCR was the same as that of the first PCR. The predicted amplified DNA products were detected in all *S. typhi* strains (Table 1 and Fig. 2B). This method also amplified the DNA from one strain of *S. paratyphi* C. It seems unlikely that this creates serious diagnostic problems, as *S. paratyphi* C is also an etiologic agent of typhoid fever. To determine the sensitivity of a nested PCR, 10-fold serial dilutions from 10^8/ml to 10^{-2}/ml of *S. typhi* GIFU10007 were prepared in saline, and DNA was extracted. As expected, the nested PCR was able to detect *S. typhi* at the single-cell level, as determined by visualization by agarose gel electrophoresis and ethidium bromide staining of the products. Thus, the nested PCR based on the ViaB sequence is a highly sensitive method of detecting *S. typhi.*

In this study, we developed a nested PCR based on the sequence encoding the Vi antigen. As expected from the limited distribution of the Vi antigen among organisms, this method is very specific for *S. typhi*. In addition, we used a nested PCR strategy, as the sensitivity of a single PCR is not enough to directly detect *S. typhi* from blood specimens (2). The combination of PCR primers based on the ViaB region and a nested-PCR strategy could offer a useful tool for rapidly and specifically detecting *S. typhi* from clinical specimens. This method is applicable for detection of *S. typhi* from all clinical specimens as well as blood specimens.

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